

(19) Japan Patent Office (JP)
(11) Japanese Unexamined Patent Application Publication
(12) Public Patent Information (A) S59 - 93100

(51) Int. Cl. ³ ID. No. Internal Filing. No. (43) Publication Date: May 29th, 1984
C 07 H 21/02 7252 - 4C
21/04 7252 - 4C

No. of Inventions: 1
Examination Request: Not Filed
(Total Pages: 9)

(54) Title of the Invention: Oligonucleotide Inducer and Manufacturing Method

(21) Application No.: S58-204306

(22) Application Filed: August 9th, 1982

(62) Patent Application: Split from S57-138136

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Specification

1. Title of the Invention: Oligonucleotide Inducer and Manufacturing Method

2. Range of Patent Claims

-1- The oligonucleotide inducer with the features shown in the formula [V] below.

[See original for chemical formula]

Formula [V]

[Where m and n are 0 or any other natural number, R¹ is a bivalent, straight chain or branched chain of hydrocarbon residue, B is a base that constitutes a nucleotide (when there are several B units, these may all be the same or different).]

-2- The oligonucleotide inducer described in Claim -1- of the range of patent claims where the base B is chosen from a group made up of adenine, thymine, cytosine and guanine.

-3- The oligonucleotide inducer described in either Claim -1- or Claim -2- of the range of patent claims where R¹ is a straight chain or a split chain alkylene group with a carbon number of 2 - 20.

-4- The oligonucleotide inducer described in any one of Claims -1- through -3- of the range of patent claims where m is a natural number from 0 to 6 and n is a natural number from 0 to 40.

-5- The method for manufacturing oligonucleotide inducers shown with the formula [V] below and having the following features. The protective groups (the protective group R² of the amino group of the 5'-terminal extension, the COR¹ group of the 3'-terminal, the base portion and the protective group of the phosphoric acid section) are all removed.

[See original for chemical formulae]

Formula [IV]

Formula [V]

[Where m and n are 0 or any other natural number, R⁰ is a substituted group, ~~normally a substituted phenyl~~

group that protects the phosphoric acid group, R^1 is a bivalent, straight chain or branched chain hydrocarbon residue, R^2 is a protective group of the amino group, the COR^4 group is a protective group of the 3'-terminal hydroxyl group, B^1 is a protected base made up of nucleotides that can be protected if necessary, B is bases that make up nucleotides. (When there are several B^1 , B or R^0 units, they may all be the same or different.

3. Detailed Description of the Invention

Background of the Invention

Technical Fields of the Invention

This invention generally pertains to new oligonucleotide inducers. More specifically, this invention pertains to oligonucleotide inducers that are made by introducing primary amino groups by means of a spacer of a suitable length onto 5'-terminal phosphoric acid group extensions of nucleotides. This invention also pertains to methods of manufacturing oligonucleotide inducers of this sort.

Prior Art

In recent years, remarkable advances have been made in the chemical synthesis of nucleic acids with the development of new condensation methods such as the insertion of new protective groups, the triester method or the phosphite method. Additionally, with the rapid progress in genetic engineering, the chemical synthesis of nucleic acids is acquiring greater significance in this field as well. For example, the production of useful substances is underway synthesizing artificial genes and using genetic manipulation (Interferon: Nature, 281, 544 (1979) and Interferon from White Blood Cells: Nature, 287, 411 (1980)). Additionally, there are examples of applications using (complementary DNA segments (primers) (Nucl. Acids Res. 8, 4057 (1980)) as matrix DNA necessary when synthesizing double-strand DNA using mRNA, reverse transcriptase from single strand DNA or DNA polymerase as probes for hybrid methods (Nucl. Acids Res. 9, 879 (1981)).

In this way, the means for synthesizing nucleic acids with organic chemicals makes it possible to synthesize oligonucleotides that have special sequences that cannot be isolated from organisms, making a great contribution to molecular biology, genetic engineering and other areas of research.

The inventors in question have been synthesizing various oligonucleotides and studying their applications. They have also studied the solid-phase method in the field of organic chemical synthesis of oligonucleotides as a powerful means of synthesis. More specifically, as a result of long-periods of great effort developing resins for use in affinity chromatography or non-radioactive affinity probes, we have discovered an oligonucleotide inducer that is an useful intermediary in their production.

Generally, it is troublesome to synthesize the oligonucleotide inducers used in resins (Arch. Biochem. Biophys., 168, 561 (1974), J. Biochem., 83, 783 (1978), JSP S52-25795, JSP S53-101396, JSP S53-133283 and JSP S55-36277, all publications) as well as the oligonucleotide inducers used in non-radioactive affinity probes (Proc. Natl. Sci. USA, 78, 6633 - 6637 (1981)) used in affinity chromatography that have been developed or have been commercially available.

In non-radioactive affinity probes, the synthesis of cytosine inducers is difficult (from the citations above) and there have been problems with difficulties synthesizing DNA that has specific, desired base sequences. Additionally, the problem of time and effort required to synthesize ligands in the literature shown below when synthesizing affinity resins has also been described.

J. Chromatog., 97, 33 (1974)

Biochem. Biophys. Acta, 304, 231 (1973)

Anal. Biochem., 71, 471 (1976)

For these reasons, useful oligonucleotide inducers have been desired for synthesizing the probes and resins described above.

Summary of the Invention

Abstract

The purpose of this invention is to supply a solution to the points described above. We will attain these goals with oligonucleotide inducers that are made by introducing functional groups (primary amino groups) that can bond just the targets with other carriers into the 5'-terminal extensions of nucleotides using spacers of an appropriate length.

So, the oligonucleotide inducers of this invention have the features shown in the formula [V] below.

Additionally, the method of manufacturing the oligonucleotide inducers of this invention shown in the formula [V] below have the following characteristics. The compounds shown in the formula [VI] below, the protective group R^2 of the amino group on the 5'-terminal extension, the COR⁴ group of the 3'-terminal, the base portion and the protective group of the phosphoric acid portion are all removed.

[See original for chemical formulae]

Formula [IV]

Formula [V]

[Where m and n are 0 or any other natural number. R^0 is a substitution group that protects the phosphoric acid group and is normally a substitution phenyl group. R^1 is a bivalent, straight chain or branched chain hydrocarbon residue, R^2 is an amino group protection group, COR⁴ is a nucleotide 3'-terminal hydroxyl group protective group, B' is a protected base that makes up the nucleotides and may be protected if necessary. B is the bases that make up a nucleotide (when there are several B, B' or R^0 units they may be identical or different).]

Effect

The oligodeoxyribonucleotides synthesized by the inventors are capable of [illegible] the shortcomings of the affinity chromatography resins described above and nucleic acid non-radioactive affinity probes. They have the following advantages.

- (a) They can manufacture the resin or probes described above having any base sequences.
- (b) Synthesis is extremely simple and mass synthesis is possible.
- (c) These have a higher (they have primary amino groups) responsiveness than other functional groups (hydroxyl groups, phosphoric acid groups and the amino groups of base portions, etc.) that exist in the oligonucleotides, so deprotected oligodeoxyribonucleotides can be used in condensation with carriers without purification. In other words, it is possible to bond selectively with amino group sections using reaction condition settings.
- (d) It is possible to immobilize oligonucleotides synthesized using any method, including solid-phase and liquid-phase methods.

Specifics of the Invention Oligonucleotide Inducers [V]

The oligonucleotide inducers of this invention are shown in formula [V] above.

In the formula, the symbol B is customarily used to indicate a deoxyribonucleoside residue without the 3'- and 5'-hydroxyls of the 2'- deoxyribonucleoside. More specifically, it has the following structure.

[see original for drawing]

The substitution group B shows the base that makes up the nucleotides, normally adenine, thymine, cytosine or guanine. If there are several Bs in the chemical compound [V], they may be either identical or different.

m and n indicate 0 or natural numbers. The reason that the degree of polymerization in the oligonucleotide inducers of this invention is expressed as $m + n$ is that in the good manufacturing method of this invention, the respective degrees of polymerization are brought about by polymerizing fractions of m and n. (See below for details.) The m in that case is effectively 0 - 6 (especially 1 - 4) and the n is effectively 0 - 40 (especially 0 - 20).

The group R^1 is a hydroxyl group of bivalent straight or branched chains that link the amino group section and the 5'-terminal phosphoric acid group of the nucleotide section of the compound [V]. This is because the alkylene groups of straight or branched chains are particularly well suited to carbon numbers of around 2 - 20. Preferably, R^1 would be an alkylene group with a carbon number of 2 - 6.

Synthesis of the Compound [V]

General Description

The compound [V], that is, the oligonucleotide inducers of this invention, can be synthesized using any [illegible] type of method.

One preferred method is to introduce through the group R^1 , a protected, primary amine group into the oligonucleotide inducer of the formula [IV] above, in other words, into the 5'-terminal phosphoric acid group. The

nucleotide base section and the phosphoric acid base portion would be protected and the hydrogen atoms of the hydroxyl groups that bond to the 3¹-terminal will be substituted with the COR⁴ groups and all of the protected groups will be removed.

At the same time, the compound in Formula [IV] can be synthesized using a method consisting of introducing a primary amine that has been protected on the 5'-hydroxyl group extension of the oligonucleotide for which the other functional group sections are protected.

Figure 1 is a flow chart showing an example of the preferred method of synthesis. The symbols used in the flow chart have the following meanings. (Those meanings or details are as described below.)

R⁰: This is a substitution group that protects the phosphoric acid group and normally, an ortho chloro phenyl group is used.

R¹: This is a bivalent straight chain or branched chain hydrocarbon residue.

R²: This is an amino group protection group and normally, a dimethoxytrityl group is used.

R³: This is a substitution base that can assign a phosphorous acid ester and is easily broken by the stable conditions of all other protection groups. Normally, cyanoethyl is used.

COR⁴: This is the protection group of the 3'-hydroxyl group that is used in the normal method of oligonucleotide synthesis. More specifically, R⁴ is a low-grade alkyl group, allyl group (especially a phenyl group or methyl substituted phenyl) or it is a carrier (polystyrene resin or polyamide resin) that has a suitable spacer used during the solid-phase synthesis method.

R⁵: This is a protection group of the 5'-terminal hydroxyl group. Normally, a methoxytrityl group is used.

m: 0 or any other natural number.

n: 0 or any other natural number.

B: Indicates a base

B': Indicates a protected base, but normally is selected from the following: N⁶- benzoyl adenine, N-isobutyl guanine, N⁶-benzoyl cytosine or thymine (In other words, protection is unnecessary.).

Synthesis of the Compound [IV]

The compound expressed in Formula [IV] can be synthesized using any method that [illegible] consist of introducing a primary amino group that has been protected, onto the 5'-hydroxyl extension of a nucleotide on which the other functional groups have been protected.

The following is a description of an embodiment (Figure 1) of a method for synthesizing the compound [IV]. In Figure 1, causing a phosphorylating agent (such as phosphor di-triazolide, phosphor dichloride or phosphor benzo-triazolide, etc.) to act on the 5'-hydroxyl group compound [0] brings about phosphorylation. Next, the amino alcohol compound [I], on which the amino group is protected. (For this compound, the amino group of the amino alkylene alcohol (NH₂ - R¹ - OH) is protected by R².) Condensation yields the compound [II].

Note that the compound [0] is an oligonucleotide and it is possible to produce it using normal methods of oligonucleotide synthesis. Methods of synthesis include the triester method, the phosphite method, their respective solid-phase and liquid-phase methods.

At the same time, it is possible to produce the compound [IV] by using a condensation agent and the compound [II] that was synthesized earlier, in the condensation of the compound [III], which was produced by hydroxylating the 5'-terminal of the compound [III] synthesized according to normal methods of synthesizing oligonucleotides or, preferably according to the solid-phase synthesis method preferred by the inventors (Tetrahedron Letters 1079, 3635 (1979), Nucleic Acids Research 8, 5473 (1980), Nucleic Acids Research 8, 5491 (1980), Nucleic Acids Research 8, 5507 (1980), Nucleic Acids Research Symposium Series 7, 281 (1980)). Condensation agents include tosyl chloride, mesitylene sulfonyl tetrazolide and mesitylene sulfonyl nitro triazolide, but mesitylene sulfonyl nitro triazolide is preferred. Note also that the reaction conditions and other details can be found in the subsequent experiment examples.

Synthesis of the Compound [V]

The compound [V] can be produced by removing all of the protective groups of the compound [IV] above. After processing the protective groups COR⁴ groups, the ortho chlorophenyl groups in the phosphate

triesters and the acyl groups in the base sections with a 0.5M tetramethyl guanine-pyridine-2-carbaldoxime dioxan-water (9:1) (v/v) solution, an alkali process (concentrated ammonia water) was used to remove them. If R^2 is a trifluoroacetyl group, the ammonia process will be sufficient to break it, but if it is an ortho nitrophenyl sulphenyl group, a mercaptoethanol process will be necessary. When using another protective group for R^2 , with a stable oligonucleotide section as a condition, it would be possible to add additional processes. Note also that many different methods of synthesizing deoxyoligoribonucleotides are already known. There are methods other than those above, depending upon the type of protective groups, their introduction or removal, condensation, etc. For more detail about general theories and [illegible] regarding the chemical synthesis of nucleic acids, please refer to "Nucleotides and Nucleotide Synthesis" (Maruzen, 1977), "Nucleic Acid Organic Chemistry" (Kagaku Dojin, 1979), "Nucleic Acid" (Asakura Publishing, 1979), Tetrahedron, 34, 31 (1978), Yugoka, 34, 723 (1978) and Chemical Region, 33, 566 (1979).

Experiment Examples

Flow Chart

The compounds (the compound (10) in the Figure 2) of this invention were manufactured according to the flow chart in Figure 2.

The symbols in Figure 2 have the following meanings.

B': Benzoylated adenine

B: Adenine

DMTr: Dimethoxytrityl

[See original for chemical formula]

(p indicates the polymerization of the polystyrene.)

R^0 : Ortho Chlorophenyl

Et: Ethyl

CE: -cyanoethyl

m: 2

n^1 : 2

n: 12

Synthesis of the Compound [V] ((10) in Figure 2)

Experiment 1 - 1

After washing 300 mg (0.033 mmol) of dimethoxytrityl adenosine/resin [(1)] (the resin is nothing more than a carrier and, in terms of appearance, the target compound that is carried by the resin is no different from resin, so hereafter, we shall refer to the compound in question that is carried by the resin as simply "the resin.") with 10 ml of an isopropanol-methylene chloride (15:85, V/V) solution three times, the resin [(2)] was produced by causing it to react in 8 ml of a zinc bromide 1.0 M isopropanol-methylene chloride solution for 5 minutes, 4 times (detritylation). The resin [(2)] was washed 3 times in 10 ml of an isopropanol-methylene chloride solution and after adding 150 mg (0.1 mmol) of a pyridine solution of dinucleotide [(3)] to this, the system was rendered anhydrous by azeotropy. 150 mg (0.5 mmol) of mesitylene sulfonyl nitro triazolide (hereafter MSNT) and 2 ml of anhydrous pyridine were added and allowed to react (condense) for 90 minutes. After the reaction, it was washed 3 times with 10 ml of pyridine. 10 ml of an anhydrous acetic acid-pyridine (1:9, (V/V)) solution containing a catalyst weight (approximately 10 mg) of dimethyl aminopyridine (hereafter DMAP) was added and allowed to react for 10 minutes. The unreacted 5'-hydroxyl groups were protected using acetylation and this was washed using pyridine, producing the compound [(4)] ($n = 2$). The above process was repeated 6 times, producing the compound [(4)] ($n = 12$).

At the same time, the 800 mg (0.71 mmol) of 5'-hydroxy-dinucleotide [(5)] and ortho chloro phenyl phosphor di-triazolide were reacted Federal Republic of 2 hours in the dioxan solution (1.0 mmol, 6 ml) of the latter. Next, 300 mg (1.4 mmol) of trifluoro acetyl - 6 - aminohexanol and 115 mg (1.4 mmol) of 1 - methyl - imidasole were added and the reaction continued for 2 hours. After the reaction was finished, the solution was distilled off and the residue was dissolved in chloroform. Washing followed in water, an aqueous solution of 0.5 M sodium dihydrogen phosphate, an aqueous solution of saturated sodium bicarbonate, and an aqueous solution of 5% sodium chloride. Drying was carried out with disodium sulfate anhydrate. After concentrating the chloroform layer, the sample was purified in a silica gel column (the eluate we used was chloroform containing 0 - 4% methanol). After concentrating the eluate, it was dripped into pentane, producing the powdered compound [(6)].

115 mg (3.45 μmol) of the compound [(4)] ($n = 12$) synthesized above was detritylated in the same manner as above, producing [(7)]. 60 mg (0.04 mmol) of the compound [(6)] was processed in 3 ml of an solution of triethyl amino-pyridine-water (1:3:1, V/V) (decyanoethylation) producing the compound [(8)]. After anhydration, 50 mg (0.2 mmol) of MSNT and 1 ml of pyridine were added and allowed to react (condense) for 90 minutes. After the reaction, the sample was washed in pyridine and methanol, dried, producing the completely protected oligonucleotide inducers [(9)].

15 mg of the oligonucleotide inducers [(9)] and 200 μl of 0.5 M tetramethyl guanidine-pyridine-2-carbaldoximate dioxan water (9:1, (V/V)) solution were added and allowed to react a room temperature for 24 hours in a centrifuge tube. After the reaction, concentrated ammonia water (2.5) ml was added and the mixture sealed to react over night at 50°C. After the reaction was finished, the product was filtered and the filtered solution concentrated. Then, after the water was broken down, an extraction was performed using ether. The water layer was concentrated and then, using a Sephadex G-50 (1.5 mm in diameter x 120 cm, eluate solution of 0.05 M bicarbonate triethyl ammonium buffering solution with a pH of 7.5), the solution was desalinated and purified, yielding the pentadeca adenylate inducer [(10)].

Additionally, the oligonucleotide inducers in experiments 1 - 2, 1 - 3, 1 - 4, 1 - 5 and 1 - 6 were all obtained using the same methods. The base sequences of the compounds from the experiments 1 - 1 ~ 1 - 6 and other information are shown in Table 1.

Table 1

Experiment	Inducer	Content of Compound (10)		
		m + n	R ¹	(B) _{m+n} B
1 - 1		14	- C ₆ H ₁₂ -	AAAAAAAAAAAAAAAA
1 - 2		14	- C ₆ H ₁₂ -	TTTTTTTTTTTTTTTT
1 - 3		11	- C ₆ H ₁₂ -	AAAAAAAAAAAAAAAA
1 - 4		13	- C ₆ H ₁₂ -	TTGGGAAGCTTCCC
1 - 5		16	- C ₅ H ₁₀ -	GGGAAGCTTTCACCTAA
1 - 6		16	- C ₅ H ₁₀ -	GGGTCGACTAACGCAGT

Where A is adenine, T is thymine, G is guanine and C is cytosine.

The results of the Experiments 1 - 1, 1 - 2 and 1 - 3 using the Sephadex and the high-speed liquid chromatography are shown in Figures 3 ~ 4, 5 ~ 6 and 7 ~ 8. Based on these results, it is clear that the compound [V] is being generated.

4. Brief Description of the Figures

Figure 1 is a flow chart showing an example of the method for synthesizing the compounds of this invention.

Figure 2 is a flow chart of the compound shown in the embodiment.

Figures 3, 5 and 7 show the elution patterns of the Sephadex G-50 for the compound [V] (for Experiments 1 - 1, 1 - 2 and 1 - 4 respectively).

Figures 4, 6 and 8 show the elution patterns of the liquid chromatography for the compound [V] (for Experiments 1 - 1, 1 - 2 and 1 - 4 respectively).

Applicant Representative: Kiyoshi INOMATA

Figure 1

[See original for figures and charts]

Figure 2

Figure 3

Figure 4

[See original for figures and charts]

Absorbance

Absorbance

Concentration (%)

Fractions

Holding Time (minutes)

Figure 5

Figure 6

[See original for figures and charts]

Absorbance

Absorbance

Concentration (%)

Fractions

Holding Time (minutes)

Figure 7

Figure 8

[See original for figures and charts]

Absorbance

Absorbance

o

Concentration (%)

Fractions

Holding Time (minutes)

[upper left side of page]

Text of Corrections Based on Stipulations of Article 17, Section 2 of the Patent Law

The following corrections have been made to Patent Application No. S58-204306 (JSP 59-93100), issued May 29, 1984 and carried in the Patent Gazette No. 59-931 as stipulated in Article 17, Section 2 of the Patent Law. 3 (2)

Int. Cl. ⁴	ID. No.	Internal Filing. No.
C 07 H 21/02		7417 - 4C
21/04		7417 - 4C

[upper right side of page]

Issue Date: December 4th, 1989
Procedural Correction Form

August 9th, 1989
[satisfactory stamp]

To: Patent Office Official: Fumitake YOSHIDA

1. Disclosure of Incident

Patent Application No. S58 - 204306

2. Title of the Invention

Oligonucleotide Inducer

3. Party Making Corrections

Relationship to Patent: Patent Applicant
Wakunaga Industries Co., Ltd.

4. Representative

Kazuhiro SATOH, Patent Attorney (6428) [illegible stamp]
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5. Date Correction Ordered

Date Sent:	Year	Month	Date
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6. Decrease in Number of Inventions due to Correction: 1

7. Objects to be Corrected

The "Title of the Invention," "Scope of Patent Claims" and "Detailed Description of the Invention" sections in the Specification.

[Patent Office Stamp (partial)]

8. Content of Corrections

(1) We correct the Title of the Invention as follows.

"Oligonucleotide Inducer"

(2) We correct the Range of Patent Claims as follows.

(3) We delete the sentences in lines 1 - 3 of the detailed description at the bottom of page 3, "This invention also pertains to, sort."

(4) We correct the sentences in lines 15 – 18 of the same document from "or non-radioactive generally synthesized" to read "...the general specificity has poor reproducibility, synthesis."

(5) Line 20, page 5, of the same document.

After the phrase, "non-radioactive affinity probe," we insert the following phrase: "(Proc. Natl. Sci. USA, 78, 6633-6637 (1981))."

(6) Lines 1 – 2 on page 6 of the same document: we delete the phrase "from the above citation."

(7) We delete lines 10 – 12 on page 6 of the same document.

(8) Lines 4 – 10 of the same document: we delete the phrase "Additionally,[IV]."

(9) Lines 3 – 4 at the bottom of page 7: we delete the phrase "R⁰ isa phenyl group."

(10) Lines 2 – 8 on page 7 of the same document: we delete the phrase "R² isprotected."

(11) Line 4 on page 8 of the same document: we correct the phrase "B', B or R⁰" to "B."

(12) Line 12 on page 8 of the same document: we correct the phrase "the above resin" to "the affinity resin."

(13) Lines 3 - 5 on page 9 of the same document: we correct the phrase "(d) solid-phase method, ...possible." as follows.

"(d) It can bond selectively, through the primary amino groups, with the carriers, biotin, hapten, enzymes, fluorescent substances, chemical light-emitting substances and other marking substances making applications on non-radioactive affinity probes and primers possible."

(14) Line 3 on page 10 of the same document: we insert the following after the phrase "one preferred method."

"The features include the following. The protective group R^2 of the amino group on the compound 5'-terminal extension, the COR^4 group of the 3'-terminal, the base section and the protective group of the phosphoric acid section expressed by the following formula [IV] are all removed."

[See original for chemical formula]

Formula [IV]

Formula [V]

Where m and n are 0 or any other natural number, R^0 is the substitution group that protects the phosphorous acid group and is normally a substituted phenyl group, R^1 is a bivalent straight chain or branched chain of hydrocarbon residue, R^2 is the protective group of an amino group, the COR^4 group is the protective group of the nucleotide 3'-terminal hydroxyl group, B' is a protected base making up nucleotides and may be protected as necessary, B is a base that makes up nucleotides (when there are several B', B and R^0 units, they may all be identical or all different.)"

In other words, this method,

(15) Lines 2 – 3 at the bottom of page 11 of the same document: we correct the phrase "dimethoxytrityl group" to read "trifluoroacetyl group."

(16) Line 11 on page 12 of the same document: we correct the term "normal metho" to read "normal dimetho."

(17) Lines 1 – 3 at the bottom of page 13 of the same document: we delete the phrase "possible methods of synthesis include....."

(18) Lines 7 – 8 on page 14 of the same document: we correct the phrase "synthesized according to hydroxylated compound" to read "synthesized the compound [III] according to..."

(19) Line 12 on page 15 of the same document: we correct "deoxyoligo" to read "oligodeoxy."

(20) Line 13 on page 19 of the same document: we correct the term "-ldoximate" to "-ldoxime"

(21) Line 2 at the bottom of page 20 of the same document: we correct the phrase "and 1 – 3" to "and "1 – 4."

Range of Patent Claims

1. An oligonucleotide inducer that has the features shown in the Formula [V], below.

[See original for chemical formula]

Formula [V]

Where m and n are 0 or any natural number, R^1 is a bivalent straight chain or branched chain of hydrocarbon residue group, B is a base that makes up nucleotides (where there are several B units, they may be identical or different.)

2. The oligonucleotide inducer described in Claim 1 of the range of patent claims where the group B may be chosen from the groups consisting of adenine, thymine, cytosine and guanine.

3. The oligonucleotide inducer described in either Claims 1 or 2 of the range of patent claims where R^1 is an alkylene group having a straight chain or a branched chain with a carbon number or 2 – 20.

4. The oligonucleotide inducer described in any of Claims 1 – 3 of the range of patent claims where m is 0 or any

Issued: December 4th, 1989

natural number up to 6, n is 0 or any natural number up to 40.